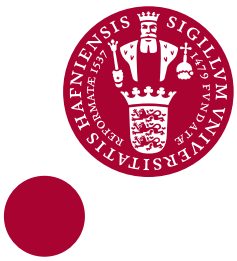

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Olofsson, Malin; Torstensson, Anders; Karlberg, Maria; Steinhoff, Franciska S.; Dinasquet, Julie Vanessa; Riemann, Lasse; Chierici, Melissa; Wulff, Angela

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Malin Olofsson*, Anders Torstensson, Maria Karlberg, Franciska S. Steinhoff, Julie Dinasquet, Lasse Riemann, Melissa Chierici and Angela Wulff

Limited response of a spring bloom community inoculated with filamentous cyanobacteria to elevated temperature and pCO_2

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Abstract: Temperature and CO_2 levels are projected to increase in the future, with consequences for carbon and nutrient cycling in brackish environments, such as the Baltic Sea. Moreover, filamentous cyanobacteria are predicted to be favored over other phytoplankton groups under these conditions. Under a 12-day outdoor experiment, we examined the effect on a natural phytoplankton spring bloom community of elevated temperature (from 1°C to 4°C) and elevated pCO_2 (from 390 to 970 µatm). No effects of elevated pCO_2 or temperature were observed on phytoplankton biovolumes, but a significantly higher photosystem II activity was observed at elevated temperature after 9 days. In addition, three species of diazotrophic filamentous cyanobacteria were inoculated to test their competitive capacity under spring bloom conditions. The toxic cyanobacterium *Nodularia spumigena* exhibited an average specific growth rate of 0.10 d^{-1} by the end of the experiment, indicating potential prevalence even during wintertime in the Baltic Sea. Generally, none of the inoculated cyanobacteria species were able to outcompete the natural phytoplankton species at temperatures ≤4°C. No direct effects were found on heterotrophic bacteria. This study demonstrates the highly efficient resistance towards short-term (12 days) changes in abiotic factors by the natural Baltic Sea spring bloom community.

Keywords: Baltic Sea; carbon dioxide; climate change; diatoms; *Nodularia spumigena*.

***Corresponding author: Malin Olofsson,** University of Gothenburg, Department of Marine Sciences, Box 461, Gothenburg SE-405 30, Sweden, e-mail: malin.olofsson@marine.gu.se
Anders Torstensson, Maria Karlberg, Franciska S. Steinhoff and Angela Wulff: University of Gothenburg, Department of Biological and Environmental Sciences, Box 461, Gothenburg SE-405 30, Sweden
Julie Dinasquet: Scripps Institution of Oceanography, UC San Diego, 9500 Gilman Drive, La Jolla, CA 92093 0202, USA; and University of Copenhagen, Marine Biological Section, Department of Biology, Strandpromenaden 5, Helsingør DK-3000, Denmark
Lasse Riemann: University of Copenhagen, Marine Biological Section, Department of Biology, Strandpromenaden 5, Helsingør DK-3000, Denmark
Melissa Chierici: Institute of Marine Research, Fram Centre, 9007, Tromsø, Norway

Introduction

The Baltic Sea covers an area of 377,000 km^2 and is the second largest brackish sea on Earth. Due to climate change, the temperature of the Baltic Sea is predicted to increase by 2–5°C over the coming 100 years (HELCOM 2013), while the global atmospheric partial pressure of CO_2 (pCO_2) will rise from current values of ca. 400 µatm (National Oceanic and Atmospheric Administration, U.S. Department of Commerce, for December 2017) to >970 µatm (IPCC 2013). Uptake of anthropogenic CO_2 is causing changes in the marine carbonate system and will result in a CO_2-enriched and more acidic state, and a concomitant elevated temperature may alter the biogenic carbon flow. In particular, in areas of high productivity and with temperatures <20°C (Wohlers et al. 2009), increased temperatures may result in higher respiration rates and decreased net productivity rates. These changes are predicted to affect all trophic levels of the planktonic food web, and thereby nutrient and carbon cycling in areas such as the Baltic Sea (Andersson et al. 2015).

Phytoplankton have flexible strategies to cope with environmental heterogeneities, e.g. changes in light conditions and nutrient supply (Boyd et al. 2016). Baltic Sea phytoplankton communities experience large daily variation in pH, e.g. ranging from early morning values of 7.8–8.4 in the afternoon during a summer bloom (Wulff et al. 2018), similar to pH ranges measured in natural aggregates of filamentous cyanobacteria (Ploug 2008). Hence, natural phytoplankton thrive despite large fluctuations in pH and pCO_2. Diatoms and dinoflagellates usually dominate the spring bloom (March–May) in the Baltic Proper (Andersson et al. 1994, Wasmund et al. 1998, Groetsch et al. 2016). Thereafter, N_2-fixing cyanobacteria like *Aphanizomenon* sp. (A. Morren ex É. Bornet and C. Flauhault 1886 '1888'), *Nodularia spumigena* (Mertens ex Bornet and Flauhault 1888)

and *Dolichospermum* spp. ((Ralfs ex Bornet and Flauhault) P. Wacklin, L. Hoffmann and J. Komárek 2009) (Larsson et al. 2001) proliferate and contribute with a large input of fixed N_2 to the system (Klawonn et al. 2016) and act as a local supply of NH_4^+ to surrounding organisms (Ploug et al. 2011). *Aphanizomenon* sp. and *N. spumigena* may be observed in low concentrations all year round (Suikkanen et al. 2010, Wasmund 2017), with high rates of N_2-fixation by *Aphanizomenon* spp. down to 10°C (Svedén et al. 2015). Whether N_2-fixation is carried out at even lower temperatures in the Baltic Sea remains unknown, but expression of nitrogenase genes has been observed locally (Bentzon-Tilia et al. 2015) and in the Arctic (Blais et al. 2012) at temperatures close to freezing. Also, potentially toxic filamentous cyanobacteria formed winter blooms in a temperate lake system at 3°C (Wejnerowski et al. 2018). A satellite-data based study of surface temperature predicts an earlier start of the summer season in the Baltic Sea (Kahru et al. 2016), with an earlier initiation of the spring bloom (Sommer et al. 2012). This may also induce an earlier and more extensive cyanobacterial summer bloom, as warmer temperatures are predicted to favor various groups of cyanobacteria (Fu et al. 2007, Paerl and Huisman 2008, Davis et al. 2009, Karlberg and Wulff 2013).

The multitude of environmental factors and complex interactions between trophic levels affected by climate change hamper qualified predictions on the future biomass and composition of phytoplankton in the Baltic Sea Proper, and on the success of various cyanobacterial groups. To start disentangling these complex interactions, we performed a bi-factorial experiment with a microplanktonic Baltic Sea community and inoculated strains of key diazotrophic filamentous cyanobacteria. Hereby, we aimed to (1) test the resistance of a natural spring bloom community, in terms of biovolume and species composition, to increased temperature and elevated pCO_2, and (2) test the potential of diazotrophic filamentous cyanobacteria to compete with the natural spring bloom community under predicted future conditions.

Materials and methods

Experimental setup and sampling procedure

The outdoor experiment was conducted between 23 March and 4 April 2011. The water was collected at the Baltic Sea Proper monitoring station B1 on 17 March 2011 (58°49′N, 17°38′E), with a salinity of 6.6 and a sea surface temperature of ca. 1°C. The collected water was gently sieved through a 200-µm mesh to exclude large grazers. Cultures of the following diazotrophic filamentous cyanobacterial strains were obtained from the Kalmar Algae Collection, Linnaeus University, Kalmar, Sweden: *Aphanizomenon* sp. (A. Morren ex É. Bornet and C. Flauhault 1886, 1888'), *Nodularia spumigena* (Mertens ex Bornet and Flauhault 1888) and *Dolichospermum* spp. ((Ralfs ex Bornet and Flauhault) P. Wacklin, L. Hoffmann and J. Komárek 2009) (KAC16, formerly known as *Anabaena* sp.), isolated from the Baltic Sea and kept at 10°C, were inoculated into the natural community. The cyanobacteria were pre-cultured for 1 week under nutrient replete conditions, 6°C and ca. 20 µmol photons m^{-2} s^{-1}. The proportion of inoculated cyanobacteria to the natural community was based on *in situ* conditions at the nearby monitoring station B1 in June with a ratio of 1:10 between cyanobacteria and diatoms + dinoflagellates (from the database of the Swedish Meteorological and Hydrological Institute, for June 2011), hereby adding ca. 0.1 mm^3 l^{-1} of the three cyanobacteria species all together. Biovolume (mm^3 l^{-1}) is recommended by HELCOM as a proxy for Baltic Sea phytoplankton biomass (Olenina et al. 2006).

After filtration and inoculation, the water was divided into 12×4-l Plexiglas aquaria. Triplicates of four treatments were established by combining ambient temperature (1°C) and elevated temperature (4°C) with ambient pCO_2 (390 µatm) and elevated pCO_2 (970 µatm) in an orthogonal design. The elevated temperature and CO_2 levels are according to the predictions by HELCOM (2013) and IPCC (2013) for the coming 100 years. The aquaria were randomly placed in two temperature controlled water baths, recorded with two loggers per basin (HOBO Pendant, Onset Computer Corporation, USA). The basins were exposed to sunlight and covered with green plastic mesh to reduce direct solar irradiance including ultraviolet radiation, mimicking photosynthetically active radiation (PAR, 400–700 nm) at 1 m water depth at station B1. PAR was measured with a LI-1000 datalogger equipped with a Li-Cor UWQ5201 PAR sensor (Li-COR, Lincoln, USA). A Li-COR radiometer equipped with a 2π PAR sensor was used to record irradiances under the mesh throughout the experiment.

The two pCO_2 treatments were established by connecting each aquarium with a tube, constantly providing synthetic air (AGA Gas, Linköping, Sweden). The gas was dispersed to the water by plastic air diffusers at a flow rate of ~15 ml min^{-1}. The aquaria were sealed with Plexiglas lids, where small holes were maintained for gas outlet to prevent backpressure build-up. In addition, each aquarium was provided with a submerged tube connected to an external syringe, which was used to transfer subsamples from the aquaria without opening the lids and thus disturbing the pCO_2 of the headspace (ca. 5 cm).

Each of the aquaria were subsampled for phytoplankton growth and species composition, bacterial abundance and production, dissolved inorganic nutrients, and carbonate system parameters at around 08:00 h on days 0, 3, 6, 9 and 12. On days 0 and 12, samples for photosynthetic pigment analysis and stoichiometry were collected. After analysis of *in situ* conditions, additional PO_4^{3-} of ca. 4 µM of was added at day 1 to avoid limitation. For each of the four treatments, one additional aquarium containing only 0.2-µm filtered seawater was sampled for pH, total alkalinity, temperature and inorganic nutrients, to control for non-organismal effects on the carbonate system.

Determination of the carbonate system

Total alkalinity (A_T) and pH were established according to Dickson et al. (2007). Samples for pH determination were 0.2-µm filtered and analyzed spectrophotometrically (Shimadzu UV-2100) at 19.0°C on the total scale (pH_T), using a 2-mM solution of m-cresol purple as indicator (Clayton et al. 1995). The pH of the indicator solution was measured and the perturbation of the sample pH by the addition of indicator was corrected according to Chierici et al. (1999).

A_T was determined using a potentiometric titration with 0.05 M HCl (Haraldsson et al. 1997). The equivalence point was estimated using Gran evaluation (Gran 1952) and manual titration with a dosimat (876 Dosimat plus, Metrohm) and a pH sensitive electrode (Aquatrode with Pt1000 Thermistor, Metrohm). The accuracy was tested using certified reference material (CRM) batch 97 supplied by A. Dickson, San Diego, USA (Dickson et al. 2007), with an average value of 2206 ± 5.3 µmol kg^{-1}.

Calculations of pCO_2, $[HCO_3^-]$, $[CO_3^{2-}]$ and $[CO_2]$ were performed with the chemical speciation model CO2SYS (Pierrot et al. 2006). Measured data for pH at 19.0°C, A_T, $[PO_4]$, $[Si(OH)_4]$, salinity, and temperature were used in the calculation together with the constant for HSO_4^- determined by Dickson (1990) and the total hydrogen ion scale (pH_T). The dissociation constants used for carbonic acid (K_1 and K_2) were determined as described by Mehrbach et al. (1973) and refitted by Dickson and Millero (1987).

Stoichiometry and analyses of dissolved inorganic nutrients

To determine the concentration of particulate organic carbon (POC) and nitrogen (particulate organic nitrogen, PON), 150-ml samples were filtered onto pre-combusted (400°C, 4 h) GF/C filters (25 mm). The filters were frozen at –20°C, freeze-dried, ground (MM301, Retsch, Haan, Germany), and analyzed on an EA 1108 (CHNS-O, Fisons Instruments, Thermo Fisher Scientific, Waltham, MA, USA) applying 2,5-bis-[5-ert.-butyl-bensoaxzol-2-yl]-thiophen as standard. To determine the concentration of dissolved inorganic $NO_3^- + NO_2^-$ (dissolved inorganic nitrogen, DIN), PO_4^{3-} (dissolved inorganic phosphorus, DIP) and Si (dissolved inorganic silicate, DISi), 20 ml of sample was filtered through a 0.2-µm pore-size filter and stored at –80°C, until analyzed within 3 weeks on an autoanalyzer (Grasshoff et al. 1999).

Photosynthetic activity

Photosynthetic activity was estimated by variable chlorophyll fluorescence measurements in photosystem II (PSII), with a WATER-PAM (Pulse amplitude modulated fluorometer) equipped with red light emitting diodes (Walz Mess- und Regeltechnik, Effeltrich, Germany). Samples were dark adapted at *in situ* temperature for a minimum of 20 min before measurements. Minimum fluorescence (F_0') was determined by applying a low level of light and the maximum fluorescence (F_m') by exposing the sample to a short saturation pulse of light (>4000 µmol photons m^{-2} s^{-1} for 0.6 s). Variable fluorescence ($F_v = F_m' - F_0'$) and maximum quantum yield (F_v/F_m) were determined for all samples. Rapid light curves (RLCs) were performed by measurement of $\Delta F/F_m'$ ($(F_m' - F_0)/F_m'$) of quasi-adapted (15 s) cells at nine levels of actinic light (0, 51, 76, 110, 165, 250, 368, 571 and 851 µmol photons m^{-2} s^{-1}) in the emitter-detector unit. Relative electron transport rate (rETR) was calculated by effective quantum yield ($\Delta F/F_m'$) multiplied by PAR irradiance. Photosynthetic parameters ($rETR_{max}$, E_k, and α_{PSII}) were calculated according to Jassby and Platt (1976), fitted by the Nelder-Mead method in the R package phytotools (Silsbe and Malkin 2015, R Core Team 2016).

Microplanktonic composition and specific growth rates

Samples (50 ml) were preserved with alkaline Lugol's solution, stored in darkness, and analyzed within 3 months using the Utermöhl method according to HELCOM (2014a). Half of the chamber was viewed at 100× magnification (Zeiss Axiovert 40CFL, Germany) and organisms >30 µm were counted and identified to species level or group (e.g. pennate diatom). Lengths and widths were measured and biovolume (mm^3 l^{-1}) was calculated according to Hillebrand et al. (1999). At 200× and 400× magnification, a

transect diagonally across the chamber bottom (1000 and 500 µm wide, respectively) was analyzed and organisms >8 µm were counted. The biovolume ($mm^3\ l^{-1}$) of organisms >8 µm was thereafter converted into POC based on Menden-Deuer and Lessard (2000). The calculated POC based on microscopy analysis was compared to the measured POC analyzed on GF/C filters, in order to estimate the fraction of organisms or organic material not identifiable by microscopy (ca. 1–8 µm). Specific growth rate was calculated for diatoms, dinoflagellates, and each cyanobacterial species separately, for days 6–12. The specific growth rate ($\mu\ d^{-1}$) = (ln *DB*–ln *DA*)/(*t*B–*t*A), where *D*A is the biovolume ($mm^3\ l^{-1}$) at the first day of a period and *DB* the biovolume at the end of the period, and *t*A is day A and *t*B is day B.

Heterotrophic bacterial abundance and productivity

1.5 ml of sample were fixed with 1% glutaraldehyde and stored at −80°C. The samples were thawed, stained with SybrGreen (Invitrogen) and counted on a FASCanto II flow cytometer (Becton Dickinson; Gasol and del Giorgio 2000) using fluorescent beads (True counts, Becton Dickinson) to calibrate the flow rate. Bacterial productivity was measured by [^{3}H]-thymidine incorporation (Fuhrman and Azam 1982) as modified for microcentrifugation by Smith and Azam (1992). Duplicate 1.7 ml aliquots were incubated with [methyl-^{3}H]-thymidine (20 nM final conc., GE Healthcare) in sterile 2.0-ml capacity polypropylene tubes for ca. 1 h at *in situ* temperature. Duplicate blanks (killed control) were prepared by adding 5% trichloracetic acid prior to the addition of isotopes. Productivity was calculated using 1.4×10^{18} cells $mole^{-1}$ thymidine incorporated (average calculated from published Baltic Sea data, SE = 0.1×10^{18} cells $mole^{-1}$ thymidine, n = 73; HELCOM 2014b) and 20 fg carbon $cell^{-1}$ (Lee and Fuhrman 1987).

Statistical analyses

All data were analyzed for effects of elevated pCO_2 and temperature by linear mixed effects (LME) modeling using the package nlme in R (R Core Team 2016, Pinheiro et al. 2017). All models were fitted using restricted maximum likelihood, where sampling day was included as a fixed, categorical factor, and aquarium was included as a random factor (random intercept) to account for repeated measurement over time. Response variables were biovolume of the cyanobacterial species, diatoms, dinoflagellates, ciliates, POC, PON, heterotrophic bacterial abundance and productivity and photosynthetic activity (F_v/F_m, $rETR_{max}$, E_k, and α_{PSII}). Multiple comparisons of significant factors were performed by Tukey's HSD test using the lsmeans package (Lenth 2016) in R. Parametric assumptions were visually verified using boxplots and Q-Q plots (Quinn and Keough 2002) and, if necessary, data were either log (POC, PON and POC:PON) or square root [cell-specific bacterial productivity (CSP), $rETR_{max}$, E_k] transformed. In addition, regression analysis was performed between bacterial abundance and biovolume of phytoplankton groups, POC:PON, POC and PON, using Pearson correlation for day 12 (SPSS). Significance was set to $p < 0.05$. Additional multivariate analyses were performed to detect differences between treatments or time on species level and are included as Supplementary material.

Results

In summary, a significantly higher F_v/F_m value was found at elevated temperatures at days 9 and 12, as compared to ambient conditions. For bacterial abundance, interaction effects between temperature and pCO_2 were found at day 12, and a higher POC:PON ratio at elevated temperatures as compared to ambient. No treatment effects on the biovolume of diatoms, dinoflagellates or filamentous cyanobacteria were observed. The results are presented in detail below.

Temperature and the carbonate system

As desired, our manipulation generated ambient and elevated temperature conditions from day 1 onwards (Figure 1A). The average pH was 7.70 in all treatments at day 0 (before starting the pCO_2 treatment), thereafter the elevated pCO_2 remained ca. 0.2 pH units below ambient pCO_2 (Table 1). The average total alkalinity (A_T) was 1527 µmol kg^{-1} at day 0, and 1573 at day 12, with no difference between the treatments (Table 1). The pCO_2 at day 12 was lower in the treatments with organisms as compared to the controls without organisms.

Stoichiometry and dissolved inorganic nutrients

There was a significant interaction between sampling day and temperature for POC and PON concentration

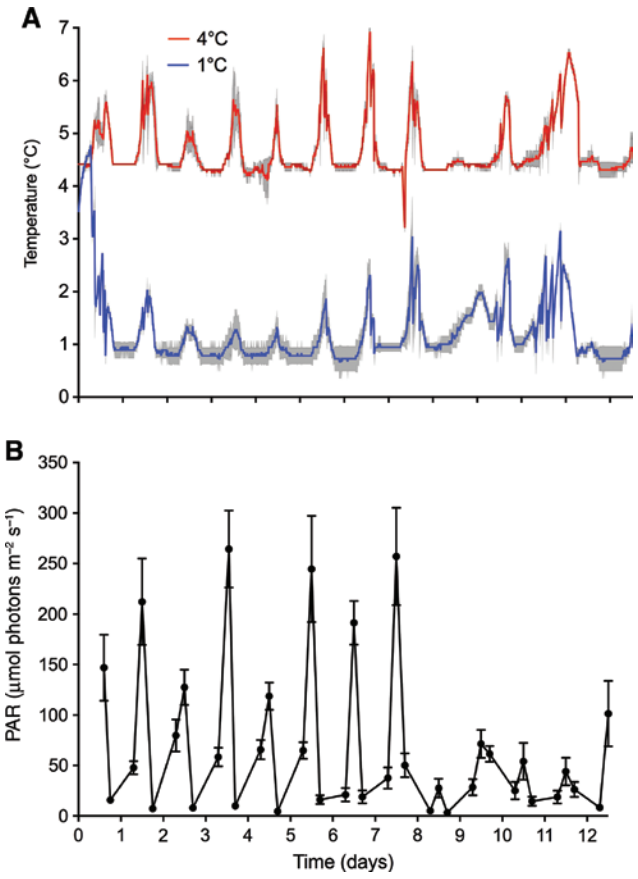

Figure 1: Environmental conditions during the experiment. (A) Temperature (°C) at ambient (blue: 1°C) and elevated (red: 4°C) conditions. (B) Radiation conditions (PAR, 400–700 nm) during the experiment. Gray areas (A) and error bars (B) indicate standard deviation, n = 2.

[p = 0.006 and 0.002, $F_{1,8}$ = 13.9 and 16.6, respectively, linear mixed effects (LME)]. The concentrations of POC and PON (Table 2) did not differ between temperatures at day 0 (p > 0.132, Tukey's test), but were significantly higher at ambient temperature as compared to elevated temperature at day 12 (p = 0.010 and p < 0.0001, respectively, Tukey's test). The measured POC (>1 µm) was ca. 20–40 times higher than the calculated POC based on microscopy (>8 µm) analysis at day 0, and ca. 46–128 times higher at day 12 (Table 2). There was a significant interaction between sampling day and pCO_2 for the POC:PON ratio (p < 0.018, $F_{1,8}$ = 8.9, LME). Thus, the interaction between sampling day and pCO_2 was reflected by a significant difference between pCO_2 treatments for POC:PON ratio at day 0 (p = 0.005, Tukey's test), but no difference at day 12 (p = 0.999, Tukey's test). Also, the POC:PON ratio was, overall, significantly higher at elevated temperature than at ambient temperature (p = 0.002, $F_{1,8}$ = 21.0, LME, Table 2).

The concentration of DIN ($NO_3^- + NO_2^-$) was around 8 µM at day 0 and decreased to below the detection limit (<0.2 µM) at elevated temperatures at day 3, but with a concentration >6 µM at ambient temperature (Figure 2A). Thereafter the concentration of DIN decreased in all treatments, to an average of 0.31 µM at day 12. The concentration of DISi (Si) remained >10 µM in all treatments throughout the experiment (Figure 2B), with lower concentrations at elevated temperature at day 12 as compared to ambient. The concentration of DIP (PO_4^{3-}) was <0.25 µM in all treatments day 0. From day 1, the concentration of PO_4^{3-} (DIP) remained >2 µM in all treatments throughout the experiment (Figure 2C). The addition of DIP changed the DIN:DIP ratio from an average of 38.66 at day 0 to an average of 0.03 at elevated temperature and 1.85 at ambient temperature at day 3 and, from day 3 and onwards, the average DIN:DIP ratio remained <0.14 in all treatments (Table 2).

Photosynthetic activity and PAR levels

There was a significant interaction between sampling day and temperature on F_v/F_m (p < 0.0001, $F_{4,32}$ = 28.9, LME). The F_v/F_m decreased from day 0 to day 6 (Figure 3A) and did not differ between temperature treatments at these sampling days (p > 0.990, Tukey's test). F_v/F_m increased from day 9, with a significantly higher ratio at increased temperature as compared to ambient at days 9 and 12 (p < 0.0001, Tukey's test). Interactions between temperature and sampling day were also observed for the RLC-derived photosynthetic parameters (Figure 3B–D), α_{PSII} (p = 0.012, $F_{3,19}$ = 4.7, LME), $rETR_{max}$ (p = 0.040, $F_{3,19}$ = 3.4, LME) and E_k (p = 0.004, $F_{3,19}$ = 6.1, LME). PAR was on average 150 µmol photons m^{-2} s^{-1} at midday during the initiation of the experiment and ranged from 100 to 250 photons m^{-2} s^{-1} on average until day 8, when PAR decreased to around 50 µmol photons m^{-2} s^{-1} until the end of the experiment (Figure 1B).

Microplanktonic composition and specific growth rates

Shannon's diversity index was significantly affected by time (p = 0.001, $F_{4,32}$ = 6.3, LME) but not by temperature and pCO_2; the index was significantly higher at day 0 compared to the following days (p < 0.01, Tukey's test). The biovolume of both centric and pennate diatoms was also significantly affected by time (p < 0.0001, $F_{4,32}$ = 77.8 and 46.5, respectively, LME). The biovolume of all diatoms

Table 1: Experimental conditions and treatments, including pH_T (presented at *in situ* temperature), temperature (°C), total alkalinity (A_T; µmol kg^{-1}) and pCO_2 (µatm).

Day	Treatment	pH_T	Temperature (°C)	A_T (µmol kg^{-1})	pCO_2 (µatm)
0	1°C 390 µatm	7.70±0.02	3.4	1520±12	802±44
	1°C 970 µatm	7.71±0.03	3.4	1529±6	804±47
	4°C 390 µatm	7.70±0.02	4.4	1537±19	813±41
	4°C 970 µatm	7.71±0.01	4.4	1520±30	797±24
	Control	7.76/7.72	4.4/3.4	1518/1507	695/758
3	1°C 390 µatm	7.81±0.01	1.0	1548±10	602±6
	1°C 970 µatm	7.60±0.04	1.0	1542±13	1002±97
	4°C 390 µatm	7.74±0.04	4.6	1546±13	746±62
	4°C 970 µatm	7.57±0.03	4.6	1576±11	1153±85
	Control	7.59/7.85	4.6/1.0	1514/1519	1012/560
6	1°C 390 µatm	7.84±0.05	0.7	1575±8	576±68
	1°C 970 µatm	7.61±0.05	0.7	1579±12	988±115
	4°C 390 µatm	7.84±0.03	4.5	1566±5	598±43
	4°C 970 µatm	7.61±0.04	4.5	1570±10	1043±98
	Control	No data	4.5/0.7	No data	No data
9	1°C 390 µatm	7.86±0.02	1.4	1557±26	539±23
	1°C 970 µatm	7.63±0.05	1.4	1567±11	933±107
	4°C 390 µatm	7.80±0.01	4.4	1574±5	669±22
	4°C 970 µatm	7.59±0.02	4.4	1569±8	1070±58
	Control	7.60/7.76	4.4/1.4	1525/1535	1156/718
12	1°C 390 µatm	7.85±0.02	0.8	1556±49	548±10
	1°C 970 µatm	7.58±0.02	0.8	1573±6	1053±50
	4°C 390 µatm	7.87±0.01	4.4	1584±9	564±14
	4°C 970 µatm	7.64±0.05	4.4	1580±5	978±119
	Control	7.60/7.78	4.4/0.8	1542/1535	1099/675

pCO_2 was derived from pH_T, A_T, temperature, salinity (6.6 throughout the experiment), inorganic phosphate and silicate concentration using CO2SYS (Pierrot et al. 2006). Data are presented for each treatment and controls (390 µatm/970 µatm for pH_T, A_T and pCO_2, and 1°C/4°C for temperature). Controls represent aquaria incubated without the addition of any microplankton. Standard deviation is included when available, n=3.

Table 2: Nutrient stoichiometry and POC:PON ratios.

Day	Treatment	POC	PON	POC:PON	DIN:DIP	POC organisms
0	1°C 390 µatm	144.5±10.1	17.3±0.9	8.4±0.2	38.8±0.9	6.1±1.0
	1°C 970 µatm	168.5±16.3	19.6±1.6	8.6±0.2	36.4±2.0	6.7±1.6
	4°C 390 µatm	176.7±43.9	20.6±4.8	8.5±0.3	41.0±3.0	6.4±1.0
	4°C 970 µatm	230.7±51.8	20.8±5.3	11.3±2.3	38.5±1.7	6.4±1.0
3	1°C 390 µatm	No data	No data	No data	1.7±0.1	3.5 ±0.6
	1°C 970 µatm	No data	No data	No data	2.0±0.2	3.9±0.7
	4°C 390 µatm	No data	No data	No data	<0.1±0.0	3.6±0.2
	4°C 970 µatm	No data	No data	No data	<0.1±0.0	3.3±0.9
6	1°C 390 µatm	No data	No data	No data	0.1±0.0	2.4±0.7
	1°C 970 µatm	No data	No data	No data	0.1±0.2	1.9±0.3
	4°C 390 µatm	No data	No data	No data	0.1±0.1	1.8±0.8
	4°C 970 µatm	No data	No data	No data	0.1±0.0	2.0±0.7
9	1°C 390 µatm	No data	No data	No data	0.09±0.02	3.0±0.7
	1°C 970 µatm	No data	No data	No data	0.10±0.08	2.2±0.5
	4°C 390 µatm	No data	No data	No data	0.12±0.01	2.4±1.3
	4°C 970 µatm	No data	No data	No data	0.09±0.01	2.0±0.7
12	1°C 390 µatm	178.0±42.6	24.9±5.2	7.1±0.5	0.1±0.1	2.9±1.3
	1°C 970 µatm	193.2±45.8	28.4±6.6	6.8±0.1	0.1±0.0	1.5±0.5
	4°C 390 µatm	127.5±25.1	15.3±3.2	8.4±0.1	0.1±0.0	2.8±0.2
	4°C 970 µatm	132.0±10.0	15.0±2.0	8.9±0.5	0.1±0.0	2.2±0.8

Particulate organic carbon (POC) and particulate organic nitrogen (PON) are presented as µM, POC:PON ratio and dissolved inorganic nitrogen:phosphate ratio (DIN:DIP) as mol:mol, and POC organisms, based on microalgal biovolume calculation from microscopy analysis, as µM. Standard deviation is included, n=3.

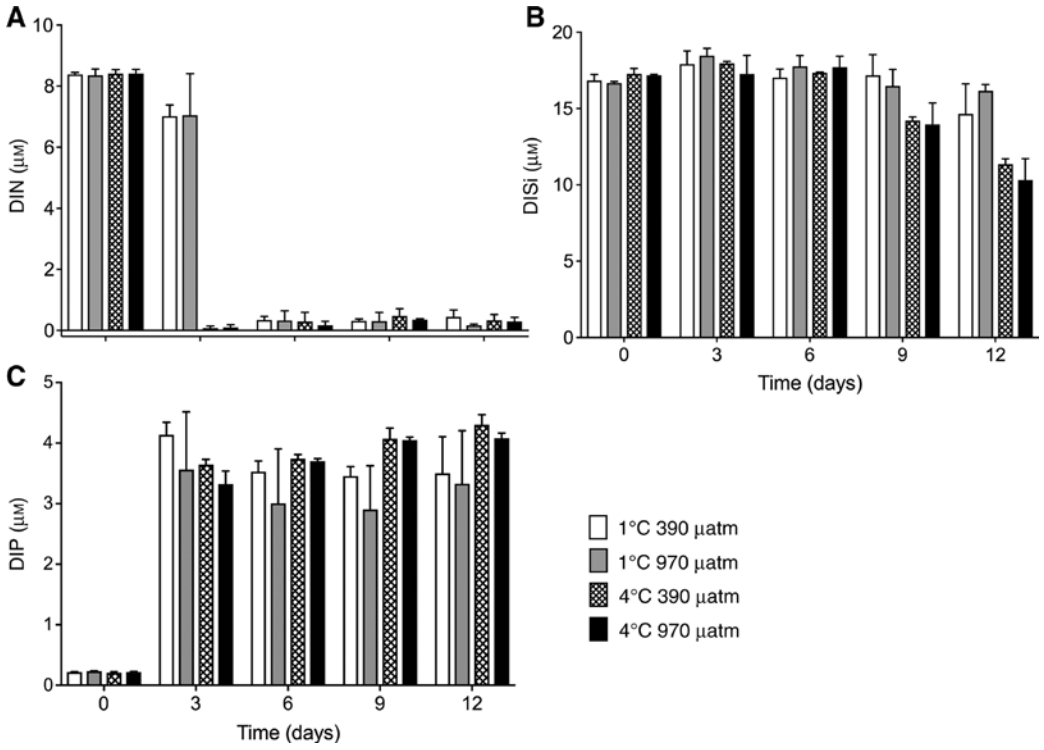

Figure 2: Inorganic nutrient concentrations in different temperature and pCO_2 treatments.
(A) Dissolved inorganic nitrogen (DIN; $NO_3^- + NO_2^-$). (B) Dissolved inorganic silica (DISi). (C) Dissolved inorganic phosphate (DIP). Error bars indicate standard deviation, n = 3.

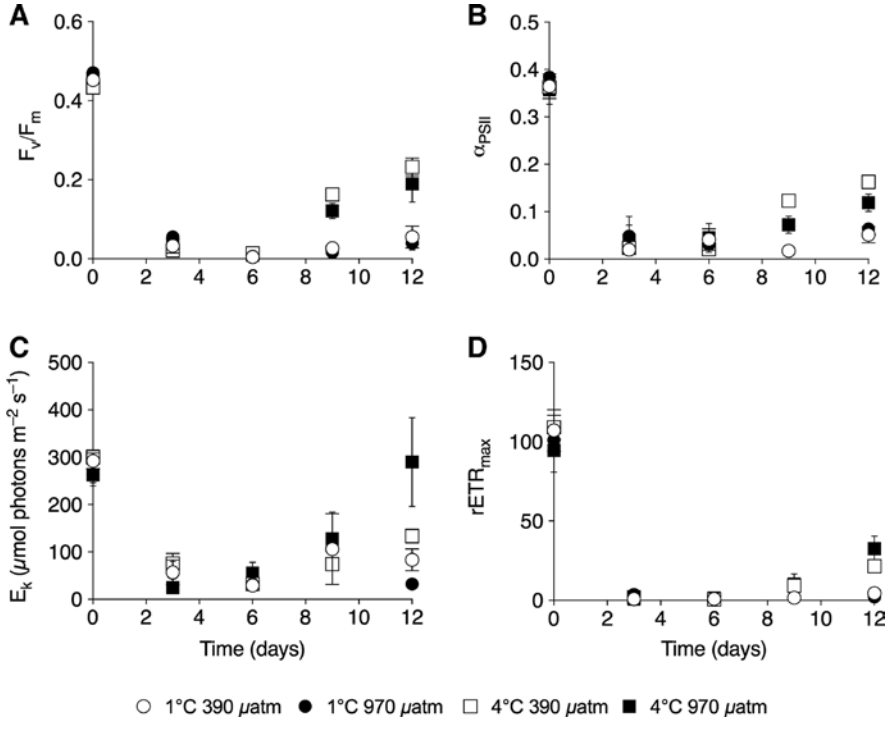

Figure 3: Photosynthetic activity of phytoplankton in different temperature and pCO_2 treatments.
(A) Maximum quantum yield: F_v/F_m. (B) Initial slope of the rapid light curve (photosynthetic efficiency): α_{PSII}. (C) Minimum saturation irradiance: E_k (µmol photons m^{-2} s^{-1}). (D) Maximum relative electron transport rate: $rETR_{max}$. Error bars indicate standard deviation, n = 3.

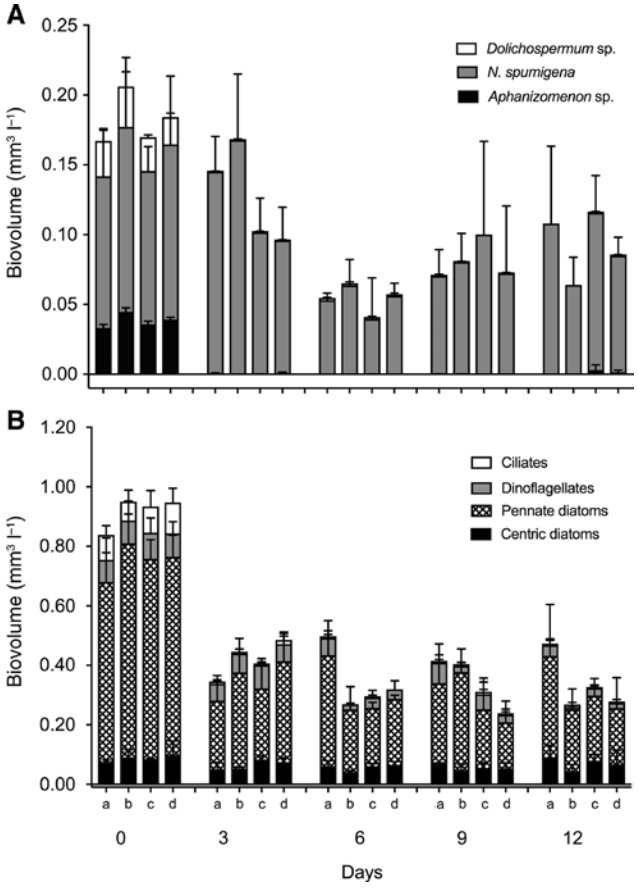

Figure 4: Biovolume (mm³ l⁻¹) of phytoplankton in different temperature and pCO_2 treatments (a: 1°C 390 µatm, b: 1°C 970 µatm, c: 4°C 390 µatm, and d: 4°C 970 µatm).
(A) Biovolume of filamentous cyanobacteria. (B) Biovolume of ciliates, dinoflagellates, pennate diatoms and centric diatoms. Error bars indicate standard deviation, n = 3.

(Figure 4B) significantly decreased between days 0 and 3 ($p < 0.0001$, Tukey's test) but then remained stable in all treatments until day 12. Neither the biovolume of pennate nor of centric diatoms showed any treatment effects at day 12 (Figure 4B). No treatment effects were found for dinoflagellates and ciliates day 12. No differences between the treatments were found for the specific growth rate of diatoms and dinoflagellates between days 6 and 12.

The biovolume of the three inoculated filamentous cyanobacteria species (*Nodularia spumigena*, *Aphanizomenon* sp. and *Dolichospermum* sp.; Figure 4A) was significantly affected by time ($p < 0.0001$, $F_{4,32} = 9.1$, 1991.6 and 148.7, respectively, LME), and significantly decreased between days 0 and 6 ($p < 0.0001$, Tukey's test). Thereafter, the biovolume of *N. spumigena* slightly increased, but there was no significant difference between treatments at day 12. *Aphanizomenon* sp. and *Dolichospermum* sp. were almost non-detectable between days 3 and 9, and cells were only occasionally found at elevated temperature at day 12. For *N. spumigena*, an average specific growth rate of 0.10 ± 0.13 d^{-1} was observed between days 6 and 12, with no significant difference between the treatments. No specific growth rate could be calculated for *Aphanizomenon* sp. and *Dolichospermum* sp. due to lack of filaments at day 6.

Heterotrophic bacterial abundance and productivity

The bacterial abundance increased for all treatments on days 0–6, followed by a decrease until day 12 (Figure 5A). The bacterial abundance was significantly affected by sampling day ($p < 0.0001$, $F_{4,29} = 312.4$, LME) and pCO_2 ($p = 0.003$, $F_{1,8} = 17.8$, LME), with an interaction effect ($p < 0.0001$, $F_{4,29} = 60.3$, LME). There were also interaction

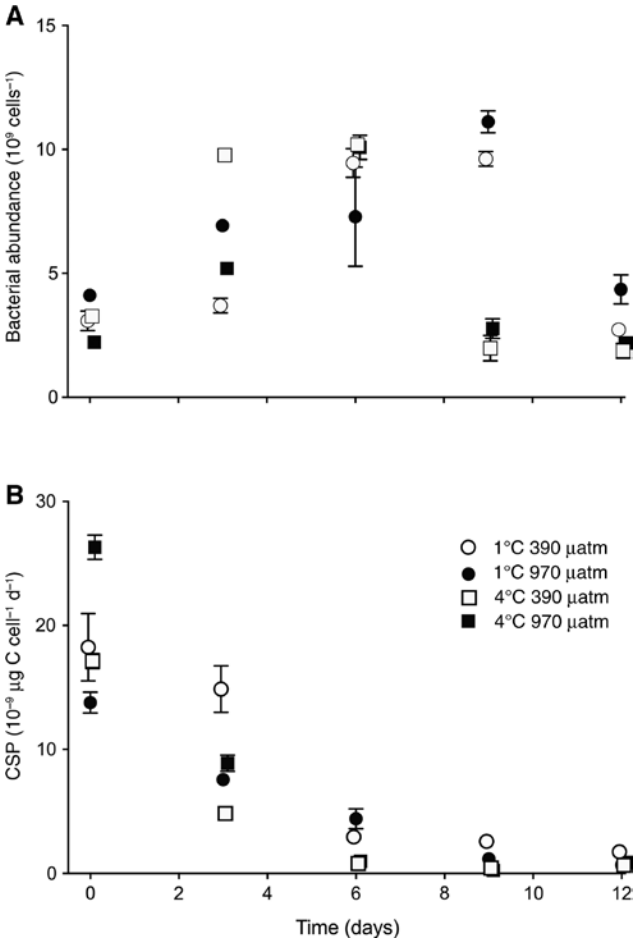

Figure 5: Heterotrophic bacterial abundance and bacterial production in different temperature and pCO_2 treatments.
(A) Bacterial abundance (10^9 cells l^{-1}). (B) Cell-specific production (CSP, 10^{-9} µg C $cell^{-1}$ d^{-1}). Error bars indicate standard deviation, n = 3.

effects for day and temperature ($p < 0.0001$, $F_{4,29} = 76.0$, LME), pCO_2 and temperature ($p < 0.0001$, $F_{1,8} = 71.0$, LME), and day, temperature and CO_2 ($p < 0.0001$, $F_{4,29} = 44.8$, LME). No significant correlations were found between bacterial abundance and groups of phytoplankton. However, significant correlations were found between bacterial abundance and POC:PON ratio ($R^2 = 0.41$, $p = 0.024$, $n = 12$), POC ($R^2 = 0.35$, $p = 0.042$, $n = 12$), and PON ($R^2 = 0.48$, $p = 0.013$, $n = 12$), with high bacterial abundance at high POC and PON concentrations and low POC:PON ratio. The cell-specific productivity (CSP; Figure 5B) was significantly affected by sampling day ($p = 0.026$, $F_{4,32} = 3.2$, LME), gradually decreasing until day 9 ($p < 0.01$, Tukey's test), and remaining stable on days 9 and 12 ($p = 0.99$, Tukey's test).

Discussion

This bi-factorial study aimed to test the resistance of a natural spring bloom community to increased temperature and elevated pCO_2. In addition, we have tested whether diazotrophic filamentous cyanobacteria, normally dominating the Baltic Sea summer bloom, can compete with the natural spring bloom species under future climate change conditions. Interestingly, neither the biovolume of the natural spring bloom community nor the inoculated filamentous cyanobacteria were affected by elevated pCO_2 or temperature conditions after being exposed for 12 days, thus, suggesting at least short-term resistance to fluctuations in the abiotic factors tested. Phytoplankton are known to have strategies to cope with environmental heterogeneities, such as changes in light conditions and nutrient supply, as they are mixed in the water column (e.g. Boyd et al. 2016). While Baltic *Nodularia spumigena* usually thrives at high temperatures and is mostly found at high densities during late summer (Suikkanen et al. 2010, Klawonn et al. 2016), we found it to be persistent and capable of growing at low temperature (≤4°C). Overall, the filamentous cyanobacteria did not outcompete the natural spring bloom species under these conditions.

Natural spring bloom community response to elevated temperature and pCO_2

All PSII parameters (F_v/F_m, α_{PSII}, $rETR_{max}$ and E_k) decreased together with the drop in DIN concentration and phytoplankton biovolume but recovered by the end of the experiment. The initial decrease in DIN concentration at elevated temperature can possibly be explained by a relatively higher affinity for NO_3^- in the phytoplankton (Reay et al. 1999). Also, the sudden drop in photosynthetic parameters suggests that the cells became nutrient stressed early on in the experiment (Beardall et al. 2001). F_v/F_m, α_{PSII}, $rETR_{max}$ and E_k were significantly higher at elevated temperatures as compared to ambient levels after 12 days, indicating that even though the total biovolume of phytoplankton was not yet affected by temperature, the cells were more active. The slow increase in photosynthetic parameters may have been triggered by regeneration of N through the decomposition of the initial standing stock of phytoplankton, or potentially from N_2-fixation by *Nodularia spumigena*. This strain of *N. spumigena* was recently shown to perform high rates of N_2-fixation in culture (Olofsson et al. 2016). From days 9 to 12 we observed a decrease in Si concentration at elevated temperature, suggesting that diatoms were the main contributor to the increase in photosynthetic activity, but this was not reflected in the biovolume (or cell numbers) of diatoms. Silica-scaled chrysophytes have been observed in the Baltic Sea, but a more likely explanation may be that diatoms attached to the walls of the aquaria were missed out in the sampling. Also, the assimilation of Si by diatoms increases with temperature, possibly explaining the decrease from day 9 to day 12 at elevated temperature (Martin-Jézéquel et al. 2000).

No short-term effects of elevated pCO_2 were observed on the biovolumes of diatoms and dinoflagellates. Previously documented short-term effects on diatoms include enhanced photosynthesis and carbon production (Wu et al. 2010, Yang and Gao 2012, Liu et al. 2017), while long-term studies have reported decreased growth rate and increased rates of respiration (7 months incubation in Torstensson et al. 2015, 1800 generations in Li et al. 2017). In a large-scale Baltic Sea mesocosm experiment, no effect on primary productivity at elevated pCO_2 (ca. 1330 µatm) was observed, while a decreased respiration rate was detected after 31 days (Spilling et al. 2016). These previous studies point at the large variation between species, incubation times and environmental conditions affecting the outcome of experimental treatments. Our study highlights the resistance to short-term fluxes by the phytoplankton community, where no major effects by elevated pCO_2 were found after 12 days. However, minor effects on species levels were detected, where the pennate diatom (10–40 µm) and the dinoflagellate *Peridiniella catenata* (Levander) Balech 1977 were negatively affected by the elevated temperature and pCO_2, respectively (Supplementary Tables S1, S2). At day 12, there were higher concentrations of POC and PON at ambient temperature as compared to elevated, which may be related to organisms

of 1–8 µm, like nano- and picoflagellates, present in the Baltic (Kuuppo 1994), which was not in focus in this study.

Response of filamentous cyanobacteria to elevated temperature and pCO_2

Filamentous cyanobacteria are important N-sources in the Baltic Sea during summer. Overall, *Aphanizomenon* sp. contributes the most to N_2-fixation due to its high biomass (Klawonn et al. 2016) and sustains high N_2-fixation rates already at 10°C during early spring (Svedén et al. 2015). In the present study, the DIN:DIP ratio was <3 from day 3 onwards, similar to late-spring conditions, which may stimulate growth of cyanobacteria (Larsson et al. 2001). Anyhow, the filamentous cyanobacteria in our study were not able to outcompete the natural spring bloom species at low temperatures. Recovery was occurring faster at higher temperature, which was interestingly not (yet) reflected in biovolume and specific growth rates. The early acclimation of PSII is most likely occurring faster than what can be observed in growth, as the light-dependent reactions generally respond rapidly and growth encompasses many more processes than photochemistry.

An earlier start of the summer season has recently been predicted in a future climate change scenario (Kahru et al. 2016). Both *Aphanizomenon* sp. and *Nodularia spumigena* are present in low concentrations during winter in the Baltic Sea, but so far very little is known about their wintertime activity (Suikkanen et al. 2010, Wasmund 2017). During winter, *Aphanizomenon* sp. appears in higher abundance than *N. spumigena* in the Baltic Sea and, thus, possibly exhibits different survival strategies in terms of initial biomass in a pre-bloom situation (Wasmund 2017). Even though the low temperature (≤4°C) is far from optimal growth temperatures for the filamentous cyanobacteria (Suikkanen et al. 2010), the increase in biomass of *N. spumigena* from day 6 onwards indicates rapid acclimation to the experimental conditions in this strain. Thus, we suggest that *N. spumigena* has a potential to grow during spring bloom conditions. The biovolume of *Aphanizomenon* sp. decreased at the initiation of the experiment, as also observed by Wulff et al. (2018), thus, no temperature effects could be observed.

Manipulations of the carbonate system using air bubbling to mimic future climate change scenarios have previously been used successfully (Gattuso et al. 2010, Karlberg and Wulff 2013, Torstensson et al. 2013, 2015, Wulff et al. 2018). A pH difference was established from day 3 onwards, even though specific pCO_2 values are difficult to control because pCO_2 also varies with photosynthesis and respiration rates (Wulff et al. 2018). Nevertheless, no effects of elevated pCO_2 were found on the filamentous cyanobacteria. These results are consistent with some previous studies (Paul et al. 2016, Wulff et al. 2018), while others show increased growth (Wannicke et al. 2012, Eichner et al. 2014). Also, filamentous cyanobacteria are known to cope with daily fluctuations in pH measured both in aggregates (Ploug 2008) and *in situ* conditions (Wulff et al. 2018).

Response of heterotrophic bacteria to elevated temperature and pCO_2

Growth of heterotrophic bacteria is limited by temperature during most of the year in the Baltic Sea (Hagström and Larsson 1984). Nevertheless, no response in bacterial abundance to higher temperature was observed in our experiment, which may indicate that in early spring an increase of 3°C would not stimulate bacterial growth. Consistent with earlier work (Burrell et al. 2017), bacterial abundance was not affected by the increase in pCO_2. The observed early increase in abundance and productivity in all treatments (up to day 6) may be due to the release of DOC from surrounding phytoplankton (Engel et al. 2013, Torstensson et al. 2015). After day 6, the decline in bacterial abundance may suggest that bacterial mortality agents (grazers or viruses) increased, especially at higher temperature. Indeed, the grazing by heterotrophic flagellates has been shown to increase with temperature in the Baltic Sea Proper (Riemann et al. 2008). In addition, other work suggests that virus-induced bacterial mortality might be particularly sensitive to temperature variations in cold environments (Vaqué et al. 2017). Hence, while we did not examine viruses and flagellates (<8 µm) in the present experiment, they may potentially play central roles for the biogeochemical cycling of nutrients and energy through the planktonic microbial loop, particularly at the low temperatures prevailing in early spring.

In conclusion, increased pCO_2 and temperature did not enhance filamentous cyanobacteria over the natural spring bloom species, but the overall higher photosynthetic activity at elevated temperatures during the latter part of the experiment indicated more active cells within the community. For the toxic *Nodularia spumigena*, a positive specific growth rate during the second half of the experiment reflects its successful acclimation to temperatures ≤4°C and indicates a putative ecological relevance also during wintertime in the Baltic Sea. Our results highlight the resistance of the natural spring bloom community to short-term variations in abiotic conditions and, as

such, suggest only limited microplankton responses to the pCO_2 and temperature levels predicted.

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Bionotes

Malin Olofsson
University of Gothenburg, Department of Marine Sciences, Box 461, Gothenburg SE-405 30, Sweden
malin.olofsson@marine.gu.se

Malin Olofsson received her PhD in Marine Sciences from the University of Gothenburg in June 2018. Her research centers on nutrient cycling associated to phytoplankton and heterotrophic bacteria. By quantifying single-cell assimilation of carbon and nitrogen using secondary ion mass spectrometry, her PhD thesis includes novel insights into nutrient uptake by single cells of phytoplankton within mixed natural communities. She has also revived 100-year-old resting stages of diatoms in order to study single-cell dynamics in nutrient demands. Future research will include further studies on phytoplankton-bacterial interactions and carbon cycling.

Anders Torstensson
University of Gothenburg, Department of Biological and Environmental Sciences, Box 461, Gothenburg SE-405 30, Sweden

Anders Torstensson is at the time of publication a postdoctoral researcher at Uppsala University, Sweden. He holds a PhD in Biology from the University of Gothenburg, Sweden, and has spent 2 years as a postdoctoral researcher at the School of Oceanography, University of Washington. His research mainly focuses on ecophysiology of sea-ice microalgae and bacteria with respect to ocean acidification and global warming.

Maria Karlberg
University of Gothenburg, Department of Biological and Environmental Sciences, Box 461, Gothenburg SE-405 30, Sweden

Maria Karlberg holds a PhD in Biology from the University of Gothenburg, Sweden. She has published 5 peer-reviewed publications mainly focused on phytoplankton ecology, with particular emphasis on bloom-forming filamentous cyanobacteria in the Baltic Sea, effects of climate change and their potential source for biofuels. Karlberg is presently employed as a plankton expert at the Swedish Meteorological and Hydrological Institute (SMHI).

Franciska S. Steinhoff
University of Gothenburg, Department of Biological and Environmental Sciences, Box 461, Gothenburg SE-405 30, Sweden

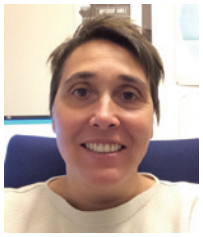
Franciska S. Steinhoff received a PhD from Bremen University, Germany in 2010 investigating the effects of global environmental changes on brown macroalgae. While she was focused on biofuel production from cyanobacteria at the University of Gothenburg, Sweden, Steinhoff has also worked in the field of industrial ecology in Trondheim, Norway. She is currently holding a position at Felleskjøpet Fôrutvikling in Norway.

Julie Dinasquet
Scripps Institution of Oceanography, UC San Diego, 9500 Gilman Drive, La Jolla, CA 92093 0202, USA
University of Copenhagen, Marine Biological Section, Department of Biology, Strandpromenaden 5, Helsingør DK-3000, Denmark

Julie Dinasquet is a postdoctoral researcher at the Scripps Institution of Oceanography, in California. She did her PhD in marine microbiology between the Linnaeus University, Sweden and Copenhagen University, Denmark. She graduated in 2013 after which she received a EU Marie-Curie postdoctoral fellowship to work between the USA in France. Her research focuses on studying the role of micro-organisms in biogeochemical cycles, atmospheric chemistry, as well as food-web interactions.

Lasse Riemann
University of Copenhagen, Marine Biological Section, Department of Biology, Strandpromenaden 5, Helsingør DK-3000, Denmark

Lasse Riemann is Professor in the Marine Biological Section, Department of Biology, University of Copenhagen, Denmark. He holds a PhD in marine microbiology from the University of Copenhagen. After doing parts of MSc/PhD at Scripps Institution of Oceanography, Riemann worked as Ass. Prof. and Assoc. Prof. at the University of Kalmar, Sweden. He returned as Assoc Prof to Univ. Copenhagen in 2010. Riemann has contributed to >90 peer-reviewed publications mainly focused on marine microbial ecology, with particular emphasis on the application of molecular tools to decipher bacterioplankton diversity, composition and functionality.



Melissa Chierici
Institute of Marine Research, Fram Centre, 9007, Tromsø, Norway

Melissa Chierici, Sr. Scientist at Institute of Marine Research (Norway) and adj. Professor at University Centre in Svalbard is an observational marine chemist with more than 20 years expertise within the carbon cycle, carbonate chemistry, and ocean acidification. Her current research focus is on the natural and anthropogenic drivers controlling the oceans shifting carbonate chemistry, and the effects of climate stressors on organisms using both field observations as well as experimental studies. This has resulted in nearly 60 peer-review articles.

Angela Wulff
University of Gothenburg, Department of Biological and Environmental Sciences, Box 461, Gothenburg SE-405 30, Sweden

Angela Wulff is Professor in Marine Ecology, Department of Biological and Environmental Sciences, University of Gothenburg, Sweden. She holds a PhD in marine ecology/marine botany from the University of Gothenburg. After a postdoc period in Denmark, Wulff returned to Gothenburg University. Wulff has contributed to >60 peer-reviewed publications mainly focused on marine ecology in cold water systems, with particular emphasis on cyanobacteria and diatoms.